

Time of Day-dependent Sorting of the Vesicular Glutamate Transporter to the Plasma Membrane*

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Neurotransmitters are concentrated into synaptic vesicles by VGLUT (vesicular glutamate transporter) or VGAT (vesicular GABA transporter). The number of VGLUTs per vesicle determines the amount of stored neurotransmitter, thereby influencing postsynaptic response. Recently, we described a strong diurnal cycling of the amount of VGLUT1 on synaptic vesicles prepared from whole mouse brain at different times of the day (Yelamanchili, S. V., Pendyala, G., Brunk, I., Darna, M., Albrecht, U., and Ahnert-Hilger, G. (2006) *J. Biol. Chem.* 281, 15671–15679). To analyze whether and how much VGLUT resides in cellular versus vesicular membranes, we developed a Pronase assay. We found that VGLUT and synaptotagmin are highly accessible to proteolytic cleavage in rat and mouse synaptosomal preparations, indicating considerable amounts of these vesicular proteins at the plasma membrane, whereas only minor amounts of synaptophysin and Rab3 are digested. Sucrose stimulation increases digestion of VGLUT, synaptotagmin, and synaptophysin due to membrane fusion that exposes the lumen-facing peptides to the extracellular space. Digestion of mouse synaptosomes prepared at different times of the day revealed a diurnal cycling of VGLUT to the plasma membrane. More VGLUT is digested at noon (Zeitgeber time 6) compared with the start of the light period (Zeitgeber time 0), whereas digestion of synaptophysin and synaptotagmin is independent of diurnal cycling. In contrast to VGLUT, the amount of VGAT appears not to vary diurnally but is decreased in membrane preparations from animals kept under constant darkness. We conclude that VGLUTs are sorted diurnally to the plasma membrane to modulate glutamate transmission during a day/night cycle, whereas VGAT expression is not oscillating but is increased in the presence of a light/dark cycle.

Synaptic vesicles store neurotransmitters and release them by Ca²⁺-dependent exocytosis upon stimulation. Transmitter

loading of synaptic vesicles is achieved by vesicular transmitter transporters that utilize the proton electrochemical gradient across the vesicle membrane as an energy source. For glutamate, the major excitatory neurotransmitter in the mammalian central nervous system, three structurally related vesicular transporters (VGLUT1–3 (vesicular glutamate transporters 1–3)) are expressed in the brain with partial overlapping expression patterns. VGLUT1 predominates in the cortex, whereas VGLUT2 is highly expressed in the diencephalon and brainstem, and VGLUT3 functions as a co-transmitter transporter in a variety of non-glutamatergic neurons (2). Studies on VGLUT1 and VGLUT2 knock-out mice revealed the amount of transporter per vesicle as being crucial for the amount of glutamate loaded and the postsynaptic response (3–5). Deletion of VGLUT1 and VGLUT2 exerts quite different phenotypical changes, which mainly reflect the different neurons and brain areas rather than regulation of neurotransmitter loading and release at the cellular level (2). For inhibitory neurotransmitters, only one transporter, VGAT (vesicular GABA transporter), has been identified that transports both γ -aminobutyric acid and glycine (6).

As all biological functions, synaptic plasticity of the various neuronal systems may change during the daily occurring light/dark cycles. Daily rhythms in different tissues including brain are orchestrated by a master clock located in the suprachiasmatic nucleus that synchronizes the physiological and biochemical pathways. At the molecular level, two transcription factors (CLOCK and BMAL1) induce transcription of the *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. In turn, the *Per* and *Cry* gene products inhibit the activity of CLOCK and BMAL1 and thereby constitute a negative feedback loop (7, 8). This molecular principle exists in almost every cell and orchestrates the circadian aspects of brain function.

We recently showed a circadian regulation of VGLUT1 expression on synaptic vesicles in the context of the day/night cycle involving *Per2* (1). This diurnal regulation appears to be modified by regulated intracellular sorting at the level of the presynaptic terminal. VGLUT1 and VGLUT2 were originally described as inorganic sodium-dependent phosphate transporters of the plasma membrane (2, 9), but the physiological aspects of this occurrence are unclear. We undertook this study to see whether VGLUT, in addition to its occurrence on synaptic vesicles, may be localized to the plasma membrane and whether a switch between these compartments may explain its time-dependent expression in synaptic vesicles. Furthermore,

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we asked whether VGAT is changed in the context of a day/night cycle. Using Pronase digestion, we found considerable amounts of VGLUT at the plasma membrane under *in vitro* conditions. This localization to the plasma membrane followed the light/dark cycle. In contrast to VGLUT, VGAT expression is independent of the time of the day but decreases under the condition of complete darkness.

EXPERIMENTAL PROCEDURES

Animals—Wistar rats were bred in the local animal house and used at the age of 3–6 months. *Per2^{Brdm1}* mice used in this study were characterized previously (10). The wild-type and *Per2^{Brdm1}* animals used represent littermates derived from intercrosses between heterozygous *Per2^{Brdm1}* mice on a 129SvEvBrd/C57/BL/6-Tyrc-Brd background. Mice were kept under either an uninterrupted 12-h light/12-h dark or dark/dark cycle for at least 7 and 3 days, respectively, before the start of the experiment (11).

Antibodies—The following antibodies were purchased from Synaptic Systems (Göttingen, Germany): mouse monoclonal antibodies against synaptophysin (12), VGLUT1 and VGLUT2 (both anti-VGLUT antibodies are isoform-specific with no cross-reactivity against VGLUT3) (13, 14), Rab3A, synaptotagmin, syntaxin, and SNAP25, and polyclonal rabbit antibodies against VGLUT1, the 116-kDa subunit of the vacuolar proton pump (H^+ -ATPase), and *N*-methyl-D-aspartate (NMDA)³ receptor-1. Polyclonal antibodies against the 55-kDa subunit (used for rat tissue) and the 110-kDa subunit (used for mouse tissue) of Na^+/K^+ -ATPase were from Upstate Signaling and Affinity Bioreagents, respectively. A monoclonal antibody against the mitochondrial protein TOM20 was purchased from Abcam (Cambridge, United Kingdom). Secondary antibodies for Western blot detection (horseradish peroxidase-conjugated horse anti-mouse and goat anti-rabbit) were purchased from Vector Laboratories (Burlingame, CA).

Synaptosomes and Synaptic Vesicles—Isolated nerve terminals (synaptosomes) were prepared at 4 °C from adult rat or mouse whole brains in the presence of protease inhibitors. In some experiments, rat synaptosomes were further purified by centrifugation through a step (6, 9, and 13%) Ficoll gradient, and the fraction enriched between 9 and 13% was used for Pronase digestion (see below). Synaptic vesicles (SVs; second lysed pellet fraction) were prepared from brains as described (15, 16). Briefly, synaptosomal pellets were resuspended in 1 ml of homogenization solution, hypo-osmotically shocked by the addition of 9 volumes of sterile water containing 10 mM Hepes (pH 7.4) and protease inhibitors, and homogenized (2000 rpm, three strokes). The lysate was centrifuged at $25,000 \times g$ for 20 min, resulting in the first lysed pellet and the corresponding first lysed supernatant, which was carefully removed and centrifuged at $350,000 \times g$ for 30 min in a Beckman TLA 101.4 rotor. This last centrifugation step yielded a pellet (second lysed pellet) enriched for SVs.

For circadian studies, mice were killed at the given time points either in the 12-h light/12-h dark cycle (Zeitgeber time

(ZT)) or the dark/dark cycle (circadian time (CT)). Under appropriate conditions, wild-type and *Per2^{Brdm1}* mutants were analyzed in parallel. Protein determination was performed by the BCA method using the individual membrane fractions, and equal amounts of protein were loaded for SDS-PAGE.

For each set of experiments, membrane fractions were run in parallel, and proteins were transferred to nitrocellulose and further processed for immunodetection using the ECL detection system. ECL-processed films were scanned by video imaging, and protein bands were densitometrically quantified using the SCAN PACK 3.0 program. It was ensured that the signals were in the linear range of the ECL detection system. Quantification was performed as described (17) along with statistical analysis using paired Student's *t* test.

Pronase Digestion—Synaptosomes (200 μ g of protein) were treated with Pronase (50 μ g/ml; Calbiochem) in sodium buffer (10 mM glucose, 5 mM KCl, 140 mM NaCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4 , and 20 mM Hepes (pH 7.4)) for 30 min at 37 °C with shaking (30 rpm) if not stated otherwise. Pronase was removed by centrifugation at $8000 \times g$ for 5 min. In some control experiments, rat synaptosomes were permeabilized using streptolysin O (SLO) (18) or extracted with Triton X 100 prior to Pronase digestion.

Stimulation of Synaptosomes—Rat or mouse synaptosomes (200 μ g) were stimulated with 0.5 M sucrose in Krebs-Ringer Hepes buffer (140 mM NaCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4 , 10 mM glucose, and 20 mM Hepes (pH 7.4)) for 15 min at 37 °C with shaking (300 rpm). Stimulation was stopped by keeping synaptosomes on ice and by adding 2 volumes of Krebs-Ringer Hepes buffer. Following centrifugation, Pronase digestion was performed as described above.

Confocal Imaging—Mouse hippocampal neurons (19) were cultivated for 6–8 days and then fixed and double-stained with a combination of VGLUT1 or VGLUT2 and one of the antibodies against Na^+/K^+ -ATPase (see above). Confocal analysis was performed as described (20).

RESULTS AND DISCUSSION

The membrane compartment of SVs and the presynaptic plasma membrane are strongly separated in neurons under resting conditions. Following stimulation, SVs fuse with the plasma membrane, and their membrane may completely collapse in the presynaptic membrane. Thus, following fusion, luminal peptide chains of vesicular membrane proteins are transiently exposed to the extracellular space. Generally, these vesicular membrane patches are retrieved by endocytosis, guaranteeing the integrity of the different membrane compartments. The recently described diurnal sorting of VGLUT1 was suggested to be mediated by a circadian-dependent traffic in and out at the plasma membrane (1). If VGLUT plays a role at the plasma membrane, relatively larger amounts of these transporters compared with other vesicular proteins must be observed at least transiently during periods of altered synaptic activity.

To analyze changes in the association of vesicular proteins with the plasma membrane, Pronase digestion was performed. Pronase is a mixture of proteolytic enzymes that should preferentially cleave the peptide chains of membrane proteins facing

³ The abbreviations used are: NMDA, *N*-methyl-D-aspartate; SV, synaptic vesicle; ZT, Zeitgeber time; CT, circadian time; SLO, streptolysin O.

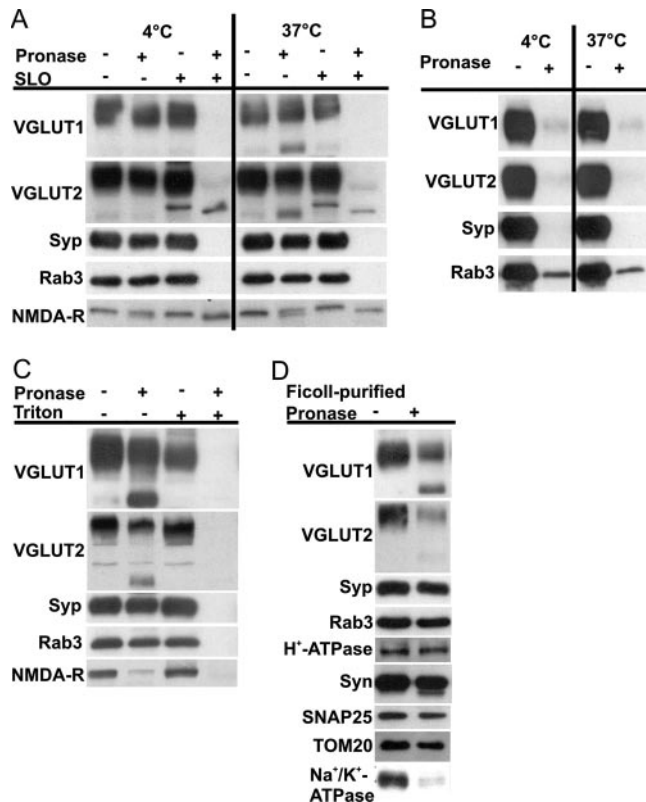


FIGURE 1. Characterization of Pronase application to synaptosomes. A, Pronase digestion of intact and permeabilized (SLO-treated) synaptosomes. Synaptosomes were prepared from rat whole brain and permeabilized with SLO followed by Pronase digestion at either 4 or 37 °C. Synaptosomal fractions were analyzed for the proteins indicated. For plasma membrane-residing proteins, better digestion was achieved at 37 °C. Note that SV proteins were completely digested by Pronase following SLO application under both conditions. B, Pronase digestion of isolated SVs. SVs were isolated from rat whole brain followed by Pronase digestion at either 4 or 37 °C. SV proteins were almost completely digested. C, Pronase digestion of intact and Triton-extracted synaptosomes. Synaptosomes were prepared from mouse whole brain and subjected to Pronase digestion at 37 °C either directly or following extraction with 1% Triton X-100, which gave complete digestion of all proteins investigated. D, Ficoll-purified rat synaptosomes subjected to Pronase digestion and analyzed for the proteins indicated. Complete digestion was observed for the Na⁺/K⁺-ATPase, whereas minor amounts of the vesicular proteins (synaptophysin (Syp) and Rab3), the plasma membrane-associated SNARE (soluble NSF attachment protein receptor) proteins (syntaxin (Syn) and SNAP25), and the mitochondrial marker TOM20 were digested. Digestion of VGLUTs was in the same range as that with crude synaptosomes. Each experiment was performed at least twice. NMDA-R, NMDA receptor.

the extraluminal or extracellular space while respecting the lipid bilayer and leaving lumen-orientated peptides protected. Pronase digestion has been successfully applied to demonstrate the orientation of the disulfide bond in the lumen-facing loops of the vesicle protein synaptophysin (21) and to estimate the lipid surface of SVs (22). When applied to synaptosomes, Pronase should also cleave the lumen-facing peptide chains of vesicular proteins sorted to the plasma membrane.

Pronase Digestion of Rat and Mouse Synaptosomes—Pronase treatment at 37 °C of rat synaptosomes resulted in partial digestion of synaptophysin and VGLUTs. The NMDA receptor was first cleaved to a peptide of lower mobility, which could be further digested (Fig. 1A; see also Fig. 1C). A complete digestion of the SV proteins Rab3, synaptophysin, VGLUT1, and VGLUT2 was observed also at 4 °C when synaptosomes were

permeabilized by SLO, which allows the proteolytic enzymes to seep into the cytosolic compartment (Fig. 1A), or when isolated SVs were directly treated with Pronase (Fig. 1B). Similarly, complete digestion was obtained when using synaptosomes extracted with Triton X-100 prior to Pronase treatment (Fig. 1C). To provide additional proof that Pronase respects the lipid bilayer and does not unspecifically disturb synaptosomal membranes, we analyzed Ficoll-purified rat synaptosomes for digestion of the VGLUTs, synaptophysin, Rab3, the H⁺-ATPase, the plasma membrane-associated proteins syntaxin and SNAP25, the extracellular facing Na⁺/K⁺-ATPase, and the mitochondrial marker protein TOM20. As shown in Fig. 1D, there was some digestion of Rab3, syntaxin, SNAP25, and TOM20. As expected, the Na⁺/K⁺-ATPase was almost completely digested. Synaptosomal preparations may be slightly contaminated by free mitochondria and SNAP25- and/or syntaxin-containing membranes derived from disrupted somata and glial cells, explaining the observed digestion. Pronase treatment and the mechanical forces from the various resuspending steps may, in addition, increase the digestion of vesicular and mitochondrial proteins due to broken synaptosomes. However, the amount of broken synaptosomes proved to be rather low in our preparations as underscored by the small amounts of digested H⁺-ATPase, Rab3, and VGAT (see also below). The data therefore indicate that Pronase cannot enter intact synaptosomes, which represent the majority in our preparations, and that unspecific cleavage of SV proteins negligibly contributes to digestion. This illustrates the efficacy of Pronase digestion of synaptosomes as an ideal tool to test for vesicular proteins at the plasma membrane of mature neurons in general.

Next, we quantified the amount of vesicular proteins such as the VGLUTs, VGAT, synaptophysin, synaptotagmin, the 110-kDa subunit of the proton ATPase, and Rab3 as well as that of some plasma membrane proteins such as the NMDA receptor and the Na⁺/K⁺-ATPase under resting conditions following Pronase digestion. We observed a digestion of 5 and 10% of the total amount of Rab3 and the proton pump, respectively, in both rat and mouse synaptosomes (Fig. 2, A and B). Because Rab3 should not be accessible for digestion, the observed decrease may be due to contaminating SVs in our preparation or to broken synaptosomes (see above). The proton pump was identified by its 115-kDa unit that has several transmembrane domains. It is present mainly on endosomal membranes, and SVs harbor only one or two copies per vesicle (22), which will not contribute much to the overall amount. So both Rab3 and the proton pump were used as internal references. Digestion of synaptophysin was not significantly higher compared with that of either Rab3 or the proton pump and reached values between 5 and 10% when subtracting these background references (Fig. 2). Using a synaptophysin-fused pHluorin, 8% of synaptophysin was calculated to reside at the plasma membrane of boutons of hippocampal neurons in culture (23), which is in the range of the average amount of synaptophysin estimated to reside on the plasma membrane using the Pronase assay. Accordingly, Pronase digestion revealed considerable amounts of synaptotagmin (25–28%) to reside on the plasma membrane under resting conditions while normalizing with Rab3 (Fig. 2). When analyzing synaptotagmin with an antibody against its luminal

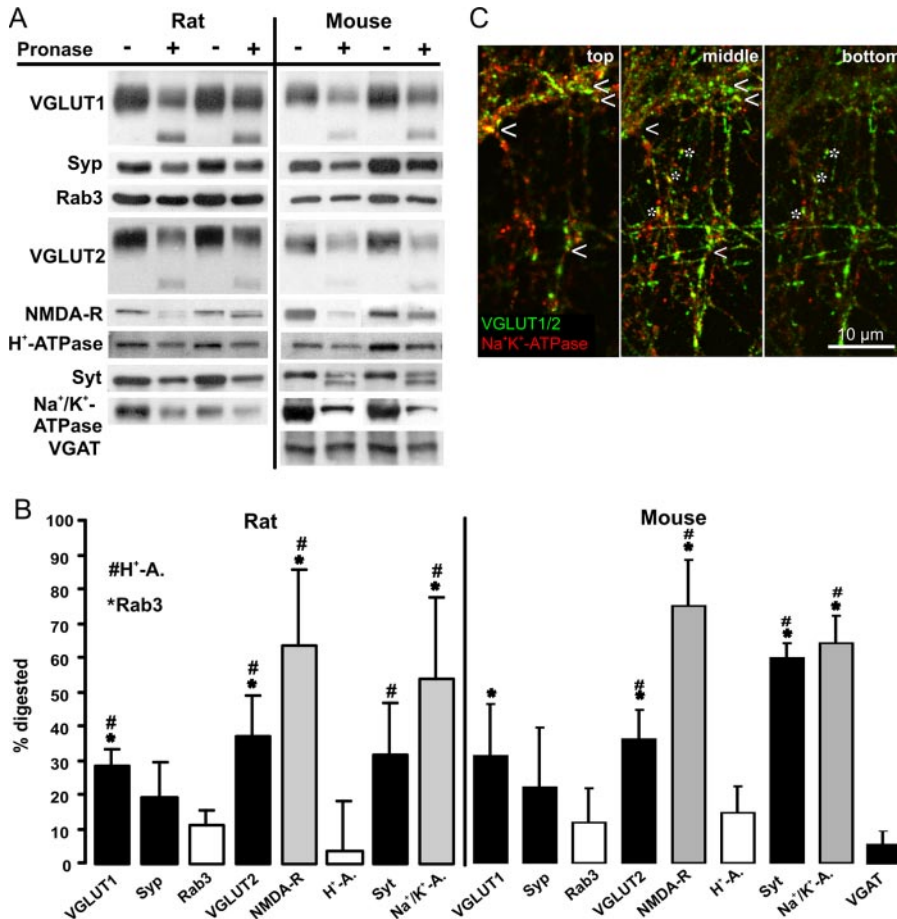


FIGURE 2. Amount of SVs and plasma membrane proteins in synaptosomes with and without Pronase digestion. A, representative gels from two different preparations of rat and mouse synaptosomes. Pronase treatment resulted in a degradation product of VGLUT1 and VGLUT2 of higher mobility. This degradation product was, however, further cleaved, as seen when SVs were directly digested (see Fig. 1B). B, amounts of digested proteins given as percent of untreated samples. Means \pm S.D. were obtained from six different experiments with either rat or mouse synaptosomes. * and #, significant ($p < 0.05$, according to Student's *t* test) digestion of proteins with respect to Rab3 and the proton pump (H⁺-ATPase (H⁺-A.)), respectively. Whereas VGLUT was digested, VGAT was resistant to Pronase treatment, indicating that it does not reside at the plasma membrane under resting conditions. C, fluorescence microscopic analysis of VGLUT and Na⁺/K⁺-ATPase in mouse hippocampal neurons reveals an occasionally occurring co-localization of the two proteins (indicated by arrowheads), suggesting the presence of VGLUT at the plasma membrane. A confocal image sectioning along the z axis was performed from the surface (top) and two subsequent 2-μm steps (middle) and (bottom) of the coverslip. The arrowheads denote double-immunopositive dots, which appear only in the top section and are reduced to VGLUT labeling in the middle section; the asterisks denote double-immunopositive dots in the middle section, which are seen only as single-labeled VGLUT spots in the bottom section. The experiment was performed twice using two different preparation of hippocampal neurons. Syp, synaptophysin; NMDA-R, NMDA receptor; Syt, synaptotagmin.

domain, ~15% of the total amount was found to be present at the plasma membrane under resting conditions in postnatal day 1–3 hippocampal neurons (24); it was also mentioned that the amounts of synaptotagmin and a synaptobrevin-fused pHluorin on the plasma membrane were comparable. In contrast, when comparing pHluorin fusion proteins of synaptophysin and synaptobrevin, the amount of synaptobrevin at the plasma membrane exceeded that of synaptophysin by 2.5–3-fold (23). Thus, the data reported for the plasma membrane pools of synaptotagmin and synaptophysin in transfected neurons are somewhat lower but in the range of the amounts found by Pronase digestion using synaptosomes from whole brain.

Considerable amounts (20–30%, corrected for the Rab3 reference) of VGLUT1 and VGLUT2 were digested (Fig. 2B). The amounts of VGLUT accessible to Pronase and thus presumed

to be present at the plasma membrane are higher than those reported in transfection studies using hippocampal neurons and VGLUT1-pHluorin (25). This may be due to the differences in the systems used, *i.e.* adult neurons from whole brain *versus* primary hippocampal neurons from embryonic brain. More important, the studies with VGLUT1-pHluorin focused on boutons and referred to transfected VGLUT1 fusion proteins in embryonic neurons, whereas Pronase digestion estimates endogenous VGLUT present in the total plasma membrane pool of a great variety of adult neurons. To determine whether endogenous VGLUT occurs at the plasma membrane under resting conditions also in early taken neurons, we analyzed the co-localization of VGLUT and the Na⁺/K⁺-ATPase in mouse hippocampal neurons. With antibodies that recognize only the cytoplasmic domains present, we performed confocal image sectioning in the z axis (surface to bottom) of the VGLUT/Na⁺/K⁺-ATPase staining. Although the majority of the VGLUT signals reflected a vesicular localization, a few double-labeled dots indicated the presence of VGLUT also at the plasma membrane (Fig. 2C, arrowheads).

VGLUT1 is developmentally up-regulated, leading to a different distribution in adult neurons in brain compared with embryonic or early taken postnatal neurons developing in culture, which may explain the higher values in our Pronase digestion assay. In addition, the good correlation between the amounts of synaptophysin and synaptotagmin obtained by both experimental approaches (see above) strengthens the data obtained for VGLUT. As expected, the plasma membrane-associated NMDA receptor and the Na⁺/K⁺-ATPase were digested by >60% (Fig. 2, A and B). The lack of complete digestion could be attributed to their presence also on endomembranes. Complete digestion was observed, however, when Pronase was applied to Triton extracts (see Fig. 1C). More remarkably, VGAT was not accessible to Pronase digestion in synaptosomal membranes, providing further confirmation of our Pronase assay (Fig. 2, A and B). In summary, Pronase digestion of synaptosomes from either rat or mouse brain reflects the overall distribution of vesicular proteins at the plasma membrane. Stimulation of synaptosomes should increase the

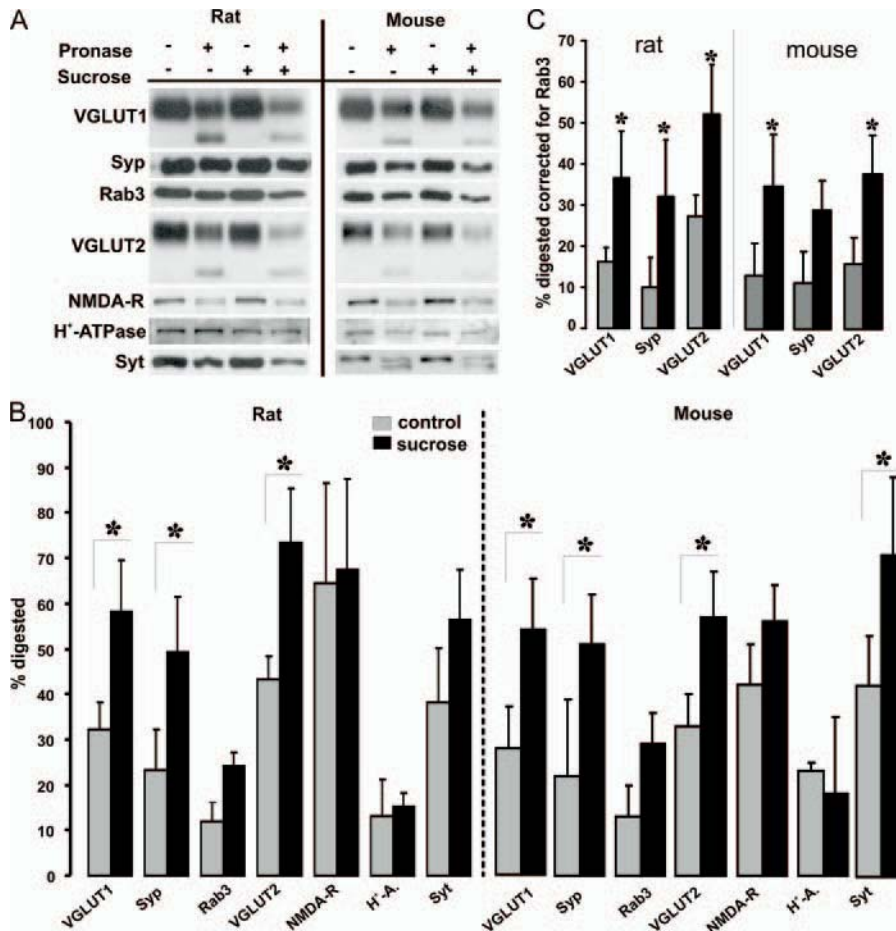


FIGURE 3. Changes in the amounts of SV proteins at the plasma membrane after sucrose stimulation. Rat or mouse synaptosomes were prepared as described under "Experimental Procedures" and incubated with or without 0.5 M sucrose prior to Pronase treatment as indicated. *A*, shown are representative gels from rat and mouse synaptosomal preparations. *B*, the amounts of digested proteins are given as percent of non-digested samples. Means \pm S.D. were obtained from four different experiments. The asterisk denotes significance ($p < 0.05$, according to Student's *t* test). Note that digestion of VGLUT1, VGLUT2, synaptophysin (*Syp*), and synaptotagmin (*Syt*) was increased after sucrose stimulation compared with control samples ($p < 0.05$). *C*, some of the data in *B* were corrected for the respective Rab3 digestion in the control and following sucrose stimulation. Significantly more VGLUT1 and VGLUT2 was digested following sucrose stimulation. *NMDA-R*, NMDA receptor.

amount of vesicular proteins appearing at the plasma membrane. Indeed, stimulation of synaptosomes from either rat or mouse brain with 0.5 M sucrose (generally used to provoke dramatic exocytotic events) increased the amount of digested synaptophysin, VGLUT1, and VGLUT2 with almost no change in the reference protein Rab3 (Fig. 3). This extensive stimulation caused considerable amounts of SVs to fuse with the plasma membrane, making the luminal domains of their transmembrane proteins accessible to Pronase digestion. Hence, an increase in the digested amounts of VGLUT1, VGLUT2, synaptophysin, and synaptotagmin was observed. When corrected for the respective Rab3 values, digestion of the VGLUTs remained significant. These experiments demonstrate that Pronase digestion enables changes in the translocation of SV proteins to the plasma membrane between resting and stimulated conditions to be tracked.

Time-dependent and Selective Appearance of VGLUT at the Plasma Membrane—Next, we tested the amount of vesicular proteins at the plasma membrane with or without digestion

in a time of day-dependent manner. Because the daily oscillations of VGLUT1 were restricted to SV preparations and not seen with synaptosomes, we speculated that VGLUT may be sorted to the plasma membrane in a time-dependent manner (1).

Synaptosomes from whole brains were prepared from wild-type mice entrained under a constant 12-h light/12-h dark cycle at the indicated time points, ZT24/0 at 6:00 a.m. (lights on) and ZT12 at 6:00 p.m. (lights off), and were immediately incubated with or without Pronase. Control and Pronase-treated synaptosomes taken at ZT24/0 and ZT6 were first analyzed for the proportional amount of digested VGLUT1, VGLUT2, synaptophysin, synaptotagmin, and Rab3. The pattern of digestion at both time points correlated with the initial control experiments (Fig. 4; see also Fig. 2), with the most digestion obtained for the plasma membrane NMDA receptor and larger amounts of VGLUT digested compared with Rab3. Interestingly, digestion of VGLUT1 and VGLUT2 was selectively enhanced at ZT6 compared with ZT24/0, whereas digestion of the NMDA receptor and the vesicular proteins synaptophysin and Rab3 remained unchanged between the time points (Fig. 4, *A* and *B*). As demonstrated before the diurnal variation, for

example, the difference between ZT0 and ZT6 in the VGLUT1/synaptophysin ratio was only seen in SVs but not in synaptosomes or homogenate, the starting material of the synaptosomal and vesicular preparations (Fig. 4C) (1). Relative amounts of vesicular proteins based on ratios to the amount of synaptophysin or Rab3 as abundant vesicle proteins were obtained. Quantification of more than six independent preparations revealed a significant decrease at ZT6 in the ratio of both VGLUT isoforms to synaptophysin or Rab3 following Pronase digestion (Fig. 5, *A* and *B*). No change was observed in the non-digested controls taken at the indicated time points and run in parallel (Fig. 5, *A* and *B*), confirming previous observations (1). In our initial experiments with SVs, expression of VGLUT1 was higher before the beginning of the light phase (ZT24/0) and the dark phase (ZT12) and lower during the middle of the day (ZT6) (1). A similar pattern was now observed using synaptosomes treated with Pronase. This indicates that the selectively reduced amount of VGLUT on SVs is due to its specific translocation to the plasma membrane. Such a translocation was

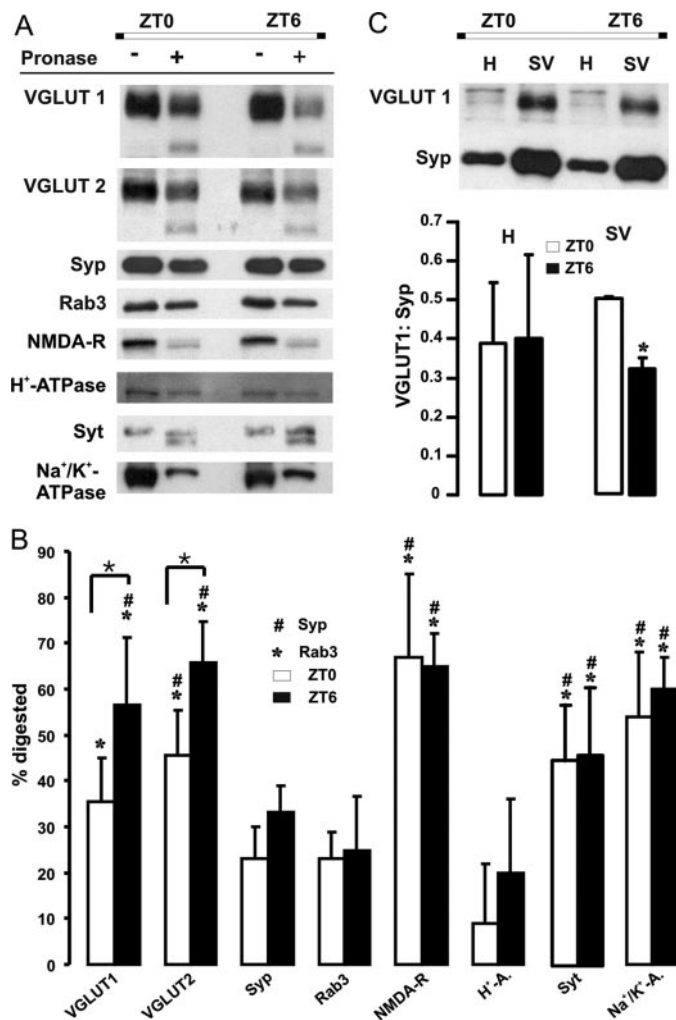


FIGURE 4. Pronase digestion of SVs and plasma membrane proteins from mouse synaptosomes prepared at different times (ZT0 and ZT6) of the day. Mice were kept under light/dark conditions. Synaptosomes were prepared at the indicated time points, with ZT0 representing dawn at 6:00 a.m., before the light was switched on, and ZT6 representing noon at 12:00 p.m., and immediately subjected to Pronase digestion as indicated. The synaptosomal fractions were analyzed for the proteins indicated. *A*, shown are representative gels from samples at ZT0 and ZT6. *B*, the amounts of digested proteins are given as percent of non-digested samples from six different animals. Values represent means \pm S.D. * and #, significant ($p < 0.05$, according to Student's *t* test) digestion of proteins with respect to Rab3 and the proton pump ($H^+-ATPase$ ($H^+-A.$)), respectively. Note that at ZT6, larger amounts of VGLUT1 and VGLUT2 were digested compared with ZT0 as indicated by the asterisks ($p < 0.05$). *C*, SVs prepared at ZT0 and ZT6 differed in the ratio between VGLUT1 and synaptophysin (*Syp*). This difference was absent when analyzing the respective homogenate (*H*). Values represent means \pm S.D. of three animals per time point. *Syt*, synaptotagmin.

absent for the SV proteins synaptophysin and synaptotagmin. This diurnal switch between the vesicle and the plasma membrane compartment applies to both VGLUT1 and VGLUT2. Synaptic terminals of glutamatergic neuronal subpopulations may differ in their cytoplasmic matrix, resulting in changes in the amount of vesicles freed by hypo-osmotic shock used under standard fractionation conditions. So it may well be that diurnal changes in the amount of VGLUT2 escaped detection in our initial analysis. Indeed, a tendency toward a diurnal oscillation has been observed (1). Because the amount of VGLUT per vesicle is crucial for transmitter filling, the observed switch may represent a diurnal change between high and low activity of

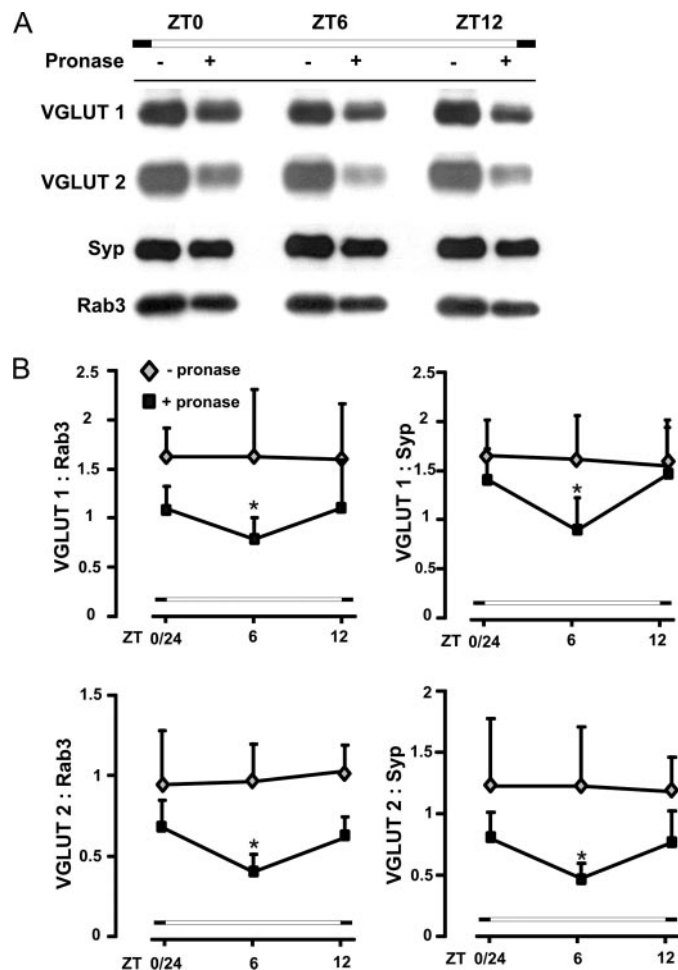


FIGURE 5. Oscillation of VGLUT amounts in Pronase-digested synaptosomes under light/dark conditions. *A*, the synaptosomes shown in Fig. 5, including preparations at ZT12 (dusk, just before the light was switched off), were analyzed for the proteins indicated. *B*, quantification was performed by calculating the ratio of synaptophysin and Rab3 to VGLUT1 and VGLUT2 in non-digested and Pronase-treated samples. Values were obtained from three different experimental sets and represent the means of four to five animals per individual time point. *, significance ($p < 0.05$, according to Student's *t* test) between ZT24/0 or ZT12 and ZT6. *Syp*, synaptophysin.

subpopulations of glutamatergic neurons. Probably the time-dependent membrane traffic allows the presynaptic terminal to replenish during physiological rest periods. How the membrane traffic of VGLUT is regulated at the presynaptic level is not clear so far. In this respect, vesicular glutamate transporters appear to be special, *i.e.* for VGLUT1, a highly specific reuptake pathway that is not shared by other vesicular proteins has been described (25).

Currently, we do not know whether VGLUT at the plasma membrane indeed represents a physiologically relevant transporter for $NaPO_4$ or only a storage pool. The interpretation is further complicated by the fact that the ionic basis of VGLUT transport activity for either $NaPO_4$ or glutamate is far from being understood. However, the occurrence of large amounts of VGLUT at the plasma membrane and the diurnal regulation of its traffic are in line with observations obtained with other transmitter transporters. Activity-dependent traffic in and out of the plasma membrane has been described for monoamine transporters such as the serotonin transporter SERT (26).

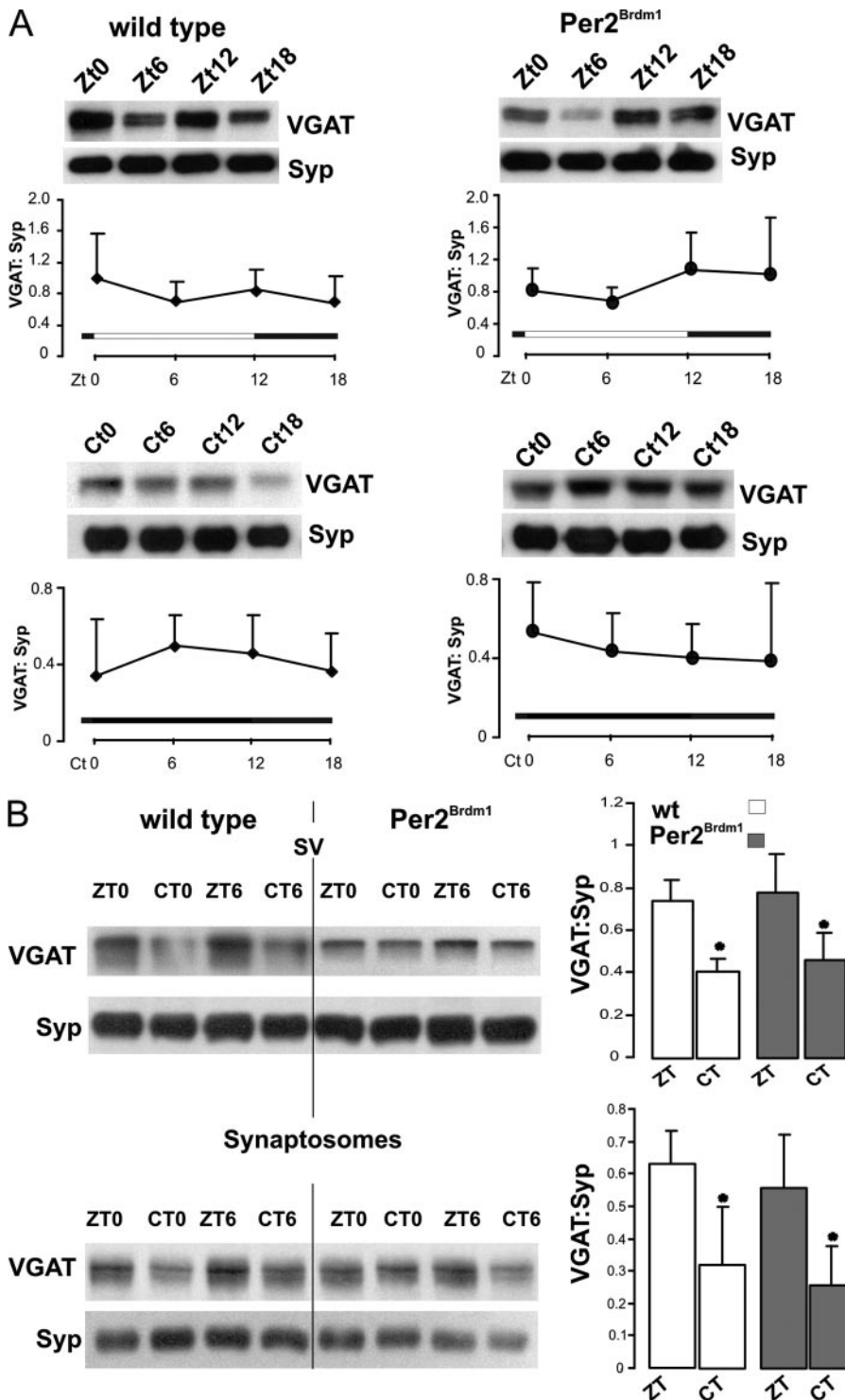


FIGURE 6. Analysis of VGAT amounts under circadian conditions. SVs or synaptosomes were prepared from wild-type or *Per2^{Brdm1}* mice kept under either light/dark (ZT) or dark/dark (CT) conditions. SVs and synaptosomes were prepared at the given times: ZT24/0 (dawn at 6:00 a.m., before the light was switched on), ZT6 (noon at 12:00 p.m.), ZT12 (dusk at 6:00 p.m., before the light was switched off), and ZT18 (night at 12:00 a.m.). A, immunoblots and quantification of the ratios of VGAT to synaptophysin (Syp) from SV preparations taken at the indicated time points from both genotypes kept under either ZT (indicated also by the white/black line) or CT (indicated also by the black line) conditions are shown. Each value represents the VGAT/synaptophysin ratio obtained from at least three animals \pm S.D. Note that the VGAT/synaptophysin ratio did not change between the various Zeitgeber or circadian time points and between the genotypes but was lower in animals kept under complete darkness. B, immunoblots directly comparing vesicular (SVs) and synaptosomal fractions prepared at two time points (ZT0/ZT6 and CT0/CT6) from wild-type (wt) and *Per2^{Brdm1}* mice are shown. Quantification was performed by calculating the ratio of synaptophysin to VGAT. Values represent the sum of all four time points (6–12 animals per condition and genotype) under either ZT or CT conditions and were obtained in three (CT) or six (ZT) sets of experiments, not all run for all the time points. *, $p < 0.05$, according to Student's *t* test.

Whereas these transporters reside on endocytotic vesicles, the choline transporter, which returns choline to the presynaptic terminal for acetylcholine synthesis, is directly localized to acetylcholine-storing SVs. This transporter is functional at the plasma membrane and appears to be inhibited when localized to SVs (27). Transmitter transporters of the plasma membrane are probably functionally linked to transporters of SVs by regulating traffic between both membrane compartments. Whether in this respect VGLUT has a dual transport function remains to be determined.

One may speculate at to how such diurnal modulation of glutamatergic transmission is achieved in the context of the whole brain. Probably at each switch, different dark/light or light/dark populations of neurons enhance their VGLUT equipment to be prepared for the specific task of the coming light or dark period, respectively. Neurons being more active just before the start of the light period may decrease their vesicular VGLUT content by sorting some copies to the plasma membrane during the middle of the day, probably before the ones responsible for the dark period tasks start to replenish their vesicular VGLUT. This explanation may also apply for the low amounts of VGLUT1 seen in the middle of the night (1).

Expression of the Vesicular GABA Transporter VGAT—The changes in the time-dependent cellular distribution of VGLUT prompted us to investigate whether VGAT may also be regulated in a time-dependent manner. There were no changes in the vesicular amount of VGAT at the indicated Zeitgeber time points, given as the VGAT/synaptophysin ratio (Fig. 6A, upper left graph). There were also no significant differences between wild-type mice and mice lacking the clock gene *Per2* (*Per2^{Brdm1}* mice) (Fig. 6A, right graphs). However, when comparing the amount of VGAT in mice entrained in a constant 12-h light/12-h dark cycle (ZT condition) with that in mice kept under complete

darkness (CT condition), the latter showed a reduction in VGAT expression. This reduction was also evident in synaptosomal preparations and was independent of the presence of *Per2* (Fig. 6, A and B). VGAT was also expressed in the absence of light; hence, its expression is not light-dependent. However, a light/dark cycle increased VGAT expression.

In conclusion, we report mechanistic evidence supporting our hypothesis that diurnal cycling in the amount of VGLUT is due to a unique sorting event that occurs between the vesicular and plasma membrane compartments. In contrast, regulation of VGAT is not mediated by a diurnal sorting mechanism in the terminal. However, light seems to be a strong signal to increase VGAT expression.

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